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14. ABSTRACT

During the first year of this project, we have made significant progress in our proposed work. We developed and tested two cell models to induce cancer dormancy: metabolic stress and Petaka incubation. We found that metabolic stress enriched quiescence population but caused spontaneous DNA breaks. Given that the goal of this project is to determine if and how DNA damage repairs play a critical role in dormancy state, metabolic stress-induced dormancy appears not to be appropriate for our study. In Petaka-induced dormancy, we found that cell dormancy facilitated DNA damage repair when cells were pre-treated with DNA damage agents, and promoted transformation phenotype and drug resistance after exited from dormant state. These observations are consistent with the fact that recurrent breast cancers are usually more malignant and more resistant to original therapy. Finally, we applied microarray analysis in cells before dormancy, maintaining in dormancy, and exited from dormancy. Further analysis of these expression profiles will help us to identify the key molecular determinants that control dormancy at different stages.

15. SUBJECT TERMS
cancer dormancy, metabolic stress, quiescence, Petaka device

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INTRODUCTION

Approximately 30% of patients with early-stage breast cancer eventually develop recurrent disease and die of breast cancer. Recurrence after treatment of early-stage breast cancer and a long period of dormancy is one of the biggest challenges in breast cancer treatment [1]. Numerous studies indicate that various mechanisms of tumor dormancy exist, including cellular dormancy (quiescence) and limit tumor size by angiogenic dormancy and immunologic dormancy [2-5]. In this award, we are focused on the role of cellular dormancy in promoting cancer aggressiveness and drug resistance in recurred breast cancer. We aim to determine the impact of dormancy on breast cancer phenotype, and to identify the molecular determinants that mediate breast cancer dormancy.

BODY

To determine cell cycle status following dormancy induction.

During the first year of this award, we established two cell models to study cellular dormancy: we applied cells with metabolic stress [6] or grew them on Petaka device at 22 °C for 14 days. Metabolic stresses were done by growing cells for 48h in the serum free media, glucose free media, or growing cells in extreme environment (serum free, contact inhibition and loss of adhesion) to enrich quiescent population (G0 phase) (Figure 1A-C). Petaka incubation was used to induce dormancy without enriching the quiescent population. As shown in Figure 1D, Petaka-induced dormancy led to accumulation of G2 phase cells without increasing total cell number. Western blotting showed that Petaka-induced dormancy reduced cyclin D1 protein levels and increased phospho-Smad (Figure 1E), two potential dormancy markers [7]. After recovered at 37 °C for 7 days, cells exited from dormancy and displayed normal cell cycle pattern and restored cyclin D1 and phospho-Smad protein levels (Figure 1F).

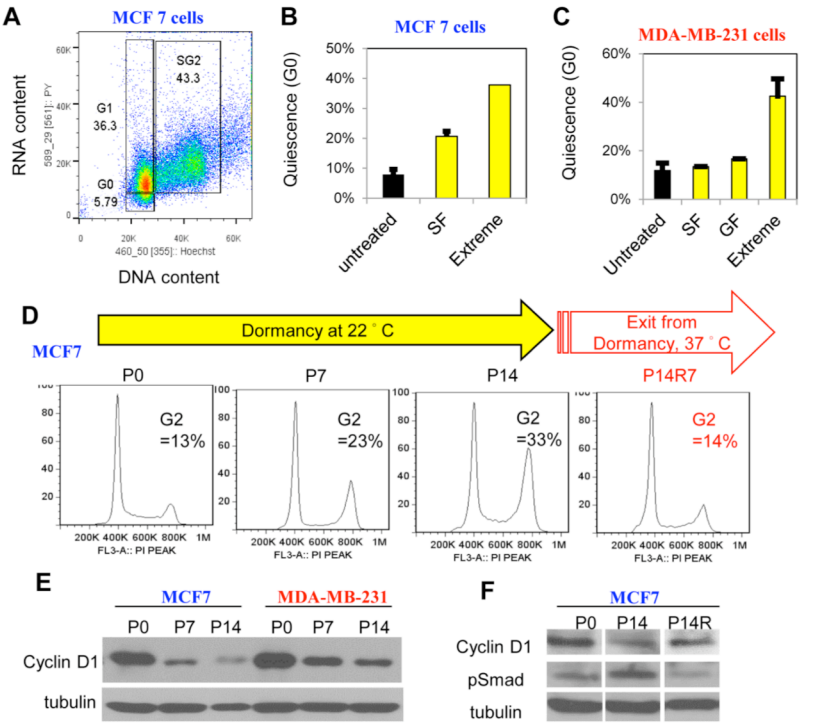

Figure 1. A. Quiescent population (G0 phase) was determined by 2N DNA content (Hoechst) and lower RNA content (Pyronin Y, PY). **B.** MCF7 and **C.** MDA-MB-231 cells were treated with metabolic stresses to enrich quiescent population. SF: serum free; GF: glucose free; Extreme: serum free, contact inhibition and loss of adhesion. **D.** DNA content analysis of MCF7 cells incubated on Petaka at 22 °C with the indicated time period; cells exited from dormancy by returning the culture temperature to 37 °C (P14R7). P0: control cells; P7: Petaka incubation for 7 days; P14: Petaka incubation for 14 days. R7: recovery for 7 days. **E.** Petaka-induced dormancy led to reduced Cyclin D1 protein levels in both MCF7 and MDA-MB231 cells. **F.** Petaka-induced dormancy reduced Cyclin D1 level and increased phospho-Smad level, two potential dormancy markers in cells. When cells exited from dormancy, both protein levels were back to normal.

To determine if cells can effectively repair damaged DNA during dormancy.

Both MCF7 and MDA-MB231 cells were pretreated with irradiation (IR, 8Gy) or doxorubicin (Doxo, 250nM for 48h) and then induced into dormancy, either by metabolic stress or Petaka incubation. After metabolic stress-induced dormancy, quiescent cells were sorted based on DNA/RNA contents described in **Figure 1A**. Comet assays using neutralization buffer were performed to determine the extent of double strand break in G0-sorted cells and Petaka-induced dormant cells (Figure 2A). As expected, following doxorubicin treatment, G0 cells had significantly increased DNA breaks. However, unexpectedly, serum free treatment alone also led to increased DNA breaks in quiescent population (Figure 2B and 2C), indicating the increase of spontaneous DNA damage in these cells. The same phenomenon was observed in cells with extreme treatment (Figure 2D). Since our goal is to study how dormant cancer cells repair DNA damage caused by the therapeutic agents such as IR or doxorubicin, the spontaneous DNA damage caused by metabolic stresses alone makes these approaches not suitable for our investigation. Therefore, all the following studies would rely on our second dormancy model by growing cells on Petaka device.

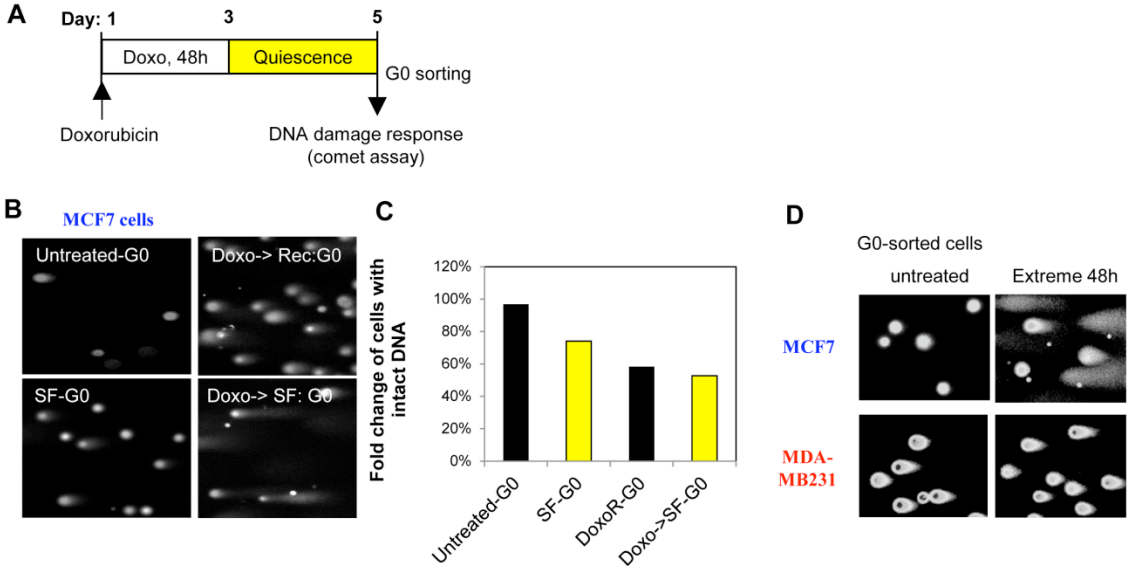

Figure 2. A. The scheme that displays the processing of comet assay in G0-sorted cells with serum free treatment. **B.** The examples of comet images of G0-sorted MCF7 cells following various treatments as the indicated. **C.** The quantitation of cells with intact DNA from comet assay in MCF7 cells following various treatments as indicated. **D**. The examples of comet images of G0-sorted cells following 48h of extreme treatment (serum free, contact inhibition and loss of adhesion).

Using our second dormancy cell model, the IR- or Doxo-pretreated MCF7 cells were induced into dormancy on Petaka device (3A). Unlike the metabolic stress model, inducing dormancy by Petaka device didn't trigger spontaneous DNA damage, making this a suitable model for our investigation. Intriguingly, as shown in Figure 3B and 3C, dormancy significantly increased cell population with intact DNA content compared to the control, indicating dormancy could facilitate DNA damage repair. To understand how dormancy promoted DNA repair, we sought to determine if dormant cells had higher non-homologous end joining (NHEJ) repair activity, a mechanism that repairs double strand breaks in non-replicating cells. Unfortunately, due to an unexpected technical difficulty, we were unable to transfect NHEJ reporter to Petaka-incubated cells. We will continue to optimize the transfection approaches to increase the uptake of the reporter gene from these dormant cells so we can assess their NHEJ activity.

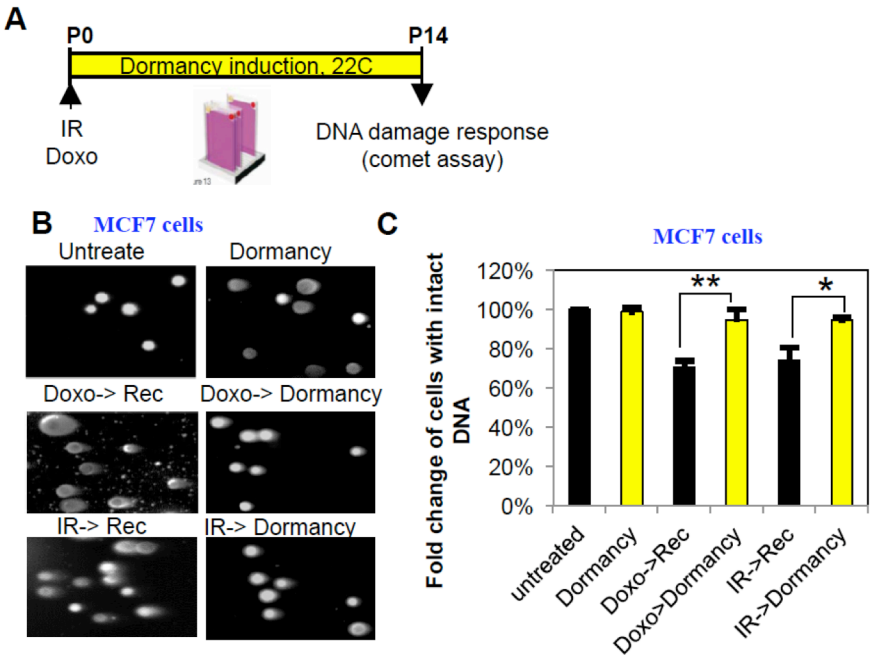

Figure 3. A. The scheme that displays the process of comet assay in Petaka-induced dormancy. Doxo: 48h treatment with 250 nM doxorubicin; IR: 8 Gy irradiation. **B.** The examples of comet images of MCF7 cells following various treatments as indicated. Rec indicated nature recovered cells without induced dormancy **C.** The quantitation of cells with intact DNA from comet assay in MCF7 cells following various treatment as indicated.*: $p<0.05$; ** $p<0.01$. Means ± SD of three independent experiments are shown.

<u>To determine if dormancy promotes more aggressive transformation phenotype and drug resistance.</u>
Since recurred breast cancers are usually more malignant than primary tumors and more resistant to original treatment, we hypothesize that the process of dormancy may enhance these characteristics. To test this hypothesis, we pretreated cells with IR or doxorubicin, inducing them into dormancy by Petaka, and then measured their transformation phenotype and their response to doxorubicin after exited from dormancy. As shown in Figure 4, cells exited from dormancy were more transformed based on their increased ability to grow on soft agar (Figure 4A) and these cells became more chemoresistant (Figure 4B).

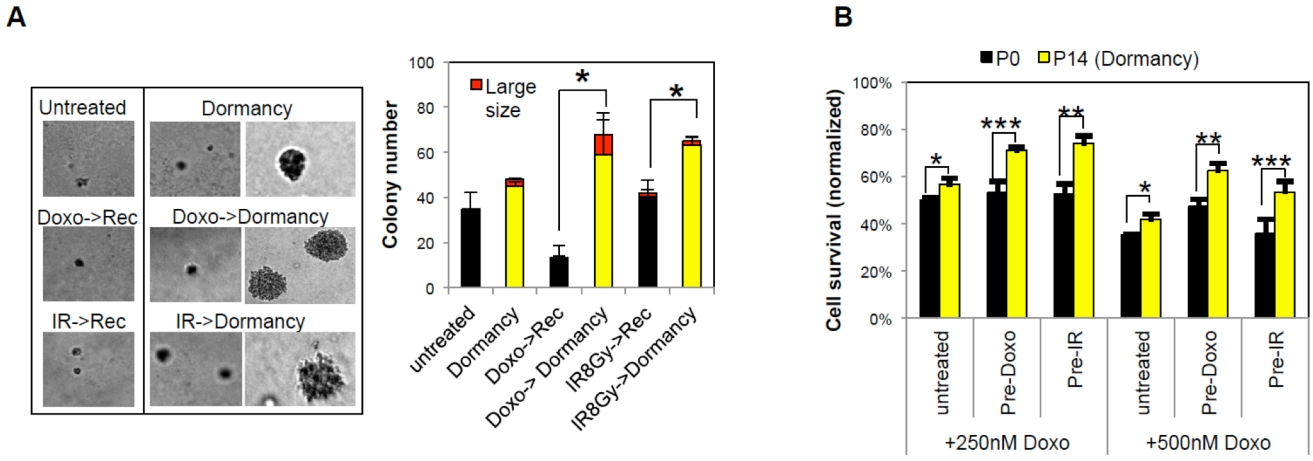

Figure 4. A. Summary of anchorage-independent growth assays of MCF7 cells that were gone through the indicated treatments. Means ± SD of three independent samples are shown. * $p<0.05$. **B.** The quantitation of cell survival of MCF7 cells as indicated following 72h of 250 nm or 500 nm doxorubicin (Doxo) treatments. Means ± SD of three independent experiments are shown. * $p<0.05$;** $p<0.01$; ***$p<0.001$.

To determine gene expression profiles of breast cancer cells at different stages of dormancy.

The transcriptoms of MCF7 cells pretreated with IR and induced to dormancy or exited from dormancy were analyzed using Illumina RNA microarray. Preliminary analysis showed that one subset of gene expression was significantly altered following Petaka-induced dormancy when compared to cells without pretreated with IR or the cells pretreated with IR but left for natural recovery (Figure 5, left panel). However, a small set of gene expression was altered between cells exited from dormancy and cells before entry of dormancy either pretreatment with IR or non-pretreatment. Further analysis will be performed to identify the candidate genes involved in all these processes and how they contribute to the more aggressive and drug resistant phenotypes in the recurred cancer.

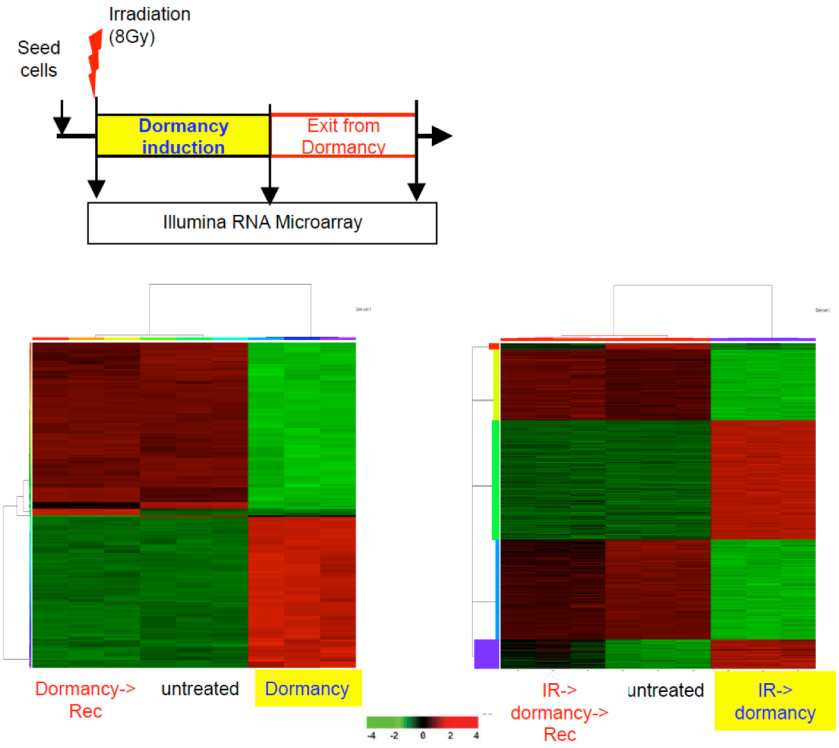

Figure 5. Preliminary analysis of microarray of RNA expression profile data from MCF7 cells following various treatments as indicated. Three independent cell samples in each treatment were applied in microarray analysis. Data are presented in matrix format; each row represents an individual gene, and each column represents a cell sample following treatment as indicated. Each cell in the matrix represents the expression level of a gene feature in treated cells. In the cells, red and green reflect relatively high and low expression levels of genes, respectively, as indicated in the scale bar (a log2-transformed scale). Two heat-map indicated one group of samples without pretreatment with IR and with pretreatment with IR.

KEY RESEARCH ACCOMPLISHMENTS

(1) We established and tested two cell dormancy models either by metabolic stress or Petaka incubation at 22 °C for 14 days.

(2) We evaluated DNA damage repair capacity in cells following dormancy induction by either metabolic stress or Petaka incubation. We concluded that the Petaka device is the method of choice to study DNA damage response in dormant cells.

(3) We demonstrated that dormancy promoted DNA-damaged cancer cells to become more transformed and more drug-resistant after their exit from dormancy. These observations are consistent with the fact that recurrent breast cancers are usually more malignant and more resistant to original therapy.

(4) We are in the process to examine the expression profiles of cells that enter into, maintain, and exit from dormancy using RNA microarray. We will identify the key molecules involved in these processes by carefully analyzing these expression profiles.

REPORTABLE OUTCOMES

Part of our study has led to an invitation of PI to present our work at Baylor College of Medicine.

CONCLUSION

In the first year of our study, we established and tested two cell models for dormancy induction. Although metabolic stresses significantly enriched quiescent population, these processes induced spontaneous DNA damage and, therefore, were not suitable for our project. Instead, we conclude that Petaka-induced dormancy can serve as a great cell model to study cancer dormancy. Using this model, we clearly demonstrated that cancer cells increased their transformation phenotype and became more drug-resistance when they had gone through the dormancy process. By analyzing the gene expression profiles of cells during this process, we expect to identify the key molecules that are involved in the process of dormancy and the mechanisms that promote cancer aggressiveness and drug resistance in the recurred breast cancer.

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